Exhibit B

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VOLUME 15 SEPTEMBER 1997

nature biotechnology

Targeted marker gene expression—in a transgenic mouse cerebellum—using a bacterial artificial chromosome modified in Escherichia coli *by homologous recombination (see p. 859). Photo courtesy Xiongdong W. Yang.*

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IN BRIEF

Business and Regulatory News

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Research News

This month in *Nature Biotechnology*

ANALYSIS

Business and Regulatory News

Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo

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Received 30 May 1997; accepted 23 July 1997

Retroviral vectors derived from lentiviruses such as HIV-1 are promising tools for human gene therapy because they mediate the in vivo delivery and long-term expression of transgenes in nondividing tissues. We describe an HIV vector system in which the virulence genes *env, vif, vpr, vpu,* and *nef* have been deleted. This multiply attenuated vector conserved the ability to transduce growth-arrested cells and monocyte-derived macrophages in culture, and could efficiently deliver genes in vivo into adult neurons. These data demonstrate the potential of lentiviral vectors in human gene therapy.

Keywords: gene therapy, retroviral vectors, HIV

Somatic gene therapy holds great promises for the treatment of a number of inherited as well as acquired disorders. Nevertheless, major obstacles remain to be overcome before these expectations are fulfilled, in particular through the development of new or improved gene delivery systems[1-4]. Retroviral vectors are appealing vehicles for gene transfer owing to several characteristics. First, they integrate their cargo into the chromosomes of target cells, a likely requisite for stable, long-term expression. Second, they do not encode viral proteins, sparing transduced cells from virus-specific immune responses. Finally, they have a relatively large capacity, allowing for the transfer of up to 10 kilobases of sequence. Until now, retroviral vectors used in clinical trials have been derived from oncoretroviruses such as the murine leukemia virus (MLV). However, these vectors can only transduce cells that divide shortly after infection, because the MLV preintegration complex cannot migrate to the nucleus in the absence of mitosis[5,6]. This considerably limits the use of MLV-based vectors for in vivo gene delivery into targets such as neurons, hepatocytes, myocytes, and hematopoietic stem cells.

Lentiviruses such as the human immunodeficiency virus type 1 (HIV-1) can infect non-proliferating cells, owing to the karyophilic properties of the lentiviral preintegration complex which govern recognition by the cell nuclear import machinery[7-10]. Correspondingly, HIV-derived vectors can transduce cell lines that are growth-arrested in culture, as well as terminally differentiated primary macrophages[11]. Furthermore, HIV-based vectors can mediate efficient delivery, integration, and sustained long-term expression of transgenes into post-mitotic cells such as adult neurons in vivo[11,12]. In spite of these advantages, a most legitimate concern about the clinical use of HIV-based vectors is linked to the possibility that the parental pathogenic virus might be reconstituted. A first line of defense against this danger is mounted by expressing the various components of the vector system from separate DNA units, so that only multiple rearrangement and recombination events could regenerate a replication competent virus. Although such occurence then becomes unlikely, it nevertheless cannot be formally excluded. By contrast, a much higher degree of biosafety would be achieved if sequences encoding critical virulence factors could be eliminated completely.

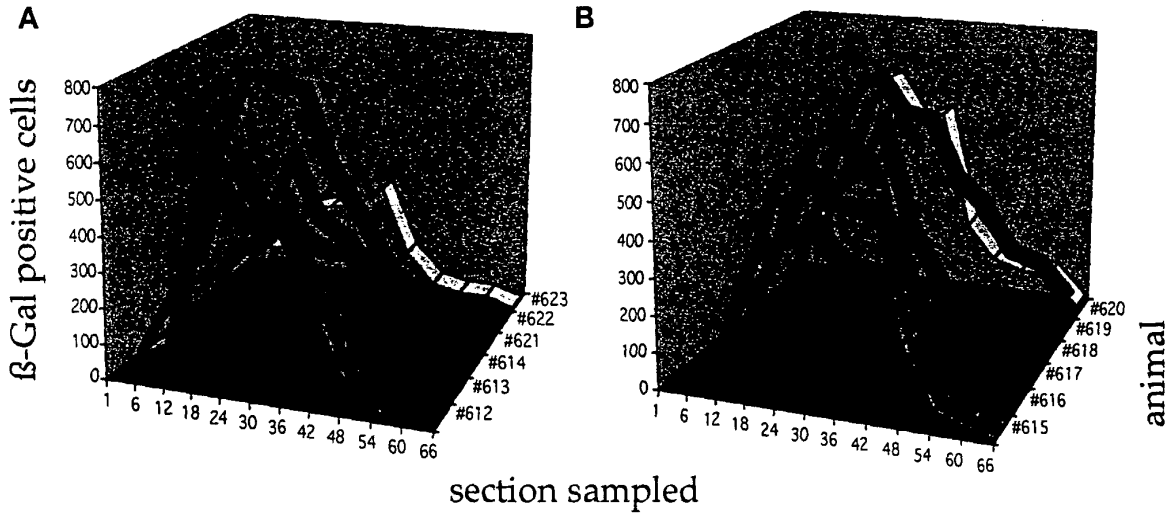
In addition to the prototypic *gag, pol,* and *env* genes found in all retroviruses, the HIV-1 genome contains six additional reading frames. Two of them, *tat* and *rev*, encode regulatory proteins which promote viral expression through transcriptional and posttranscriptional mechanisms, respectively. The other four, *vif, vpr, vpu,* and *nef* are called accessory because they can be deleted without affecting viral replication, at least in some in vitro systems. However, these genes are strictly conserved and encode proteins that are either proven or strongly suspected to represent essential virulence factors in vivo[13,14].

The *env* gene of HIV is dispensable for generating fully competent HIV-derived vector particles, as these can be pseudotyped with the surface glycoproteins of other viruses such as MLV or vesicular stomatitis virus (VSV)[11,12]. We describe an HIV-derived retroviral vector system that is further deleted in *vif, vpr, vpu,* and *nef*. This multiply attenuated vector conserved a full ability to transduce nondividing cells in vitro, including terminally differentiated macrophages. Furthermore, it mediated the in vivo delivery of transgenes into adult neurons as efficiently as a vector produced with a wild-type complement of accessory genes.

Results

Production of HIV-1-based vectors in the absence of Env, Vif, Vpr, Vpu, and Nef. The production of pseudotyped, HIV-1-based vector particles by cotransfection of three plasmids into 293T cells has been described previously[11,12]. The original system includes: (1) a packaging construct, in which the CMV immediate early promoter drives the synthesis of all HIV-1 proteins besides envelope; (2) a plasmid producing an envelope, for instance the G protein of VSV in the experiments described here; and (3) the vector itself, in which an expression cassette for the transgene is flanked by the HIV-1-derived cis-acting sequences necessary for packaging, reverse transcription, and integration. In order to identify the minimal set of HIV-1 proteins necessary for generating fully functional vector particles, the genes encoding Vif, Vpr, Vpu, and Nef were deleted from the packaging construct, either individually or in various combinations (Table 1). Retroviral particles containing a luciferase or a β-galactosidase (β-gal) reporter gene were produced from these packaging plasmids. The efficiency of viral particle production was unaffected by the deletions, even when all four accessory genes were eliminated (data not shown). This is consistent with the observation that *gag* is sufficient for this process[15].

Figure 1. In vivo transduction of brain cells by wild-type and multiply attenuated lentiviral vectors. Top: Immunohistochemical detection of β-gal activiy in transduced cells surrounding the injection site. Bottom: Quantification of β-gal positive cells on a series of brain sections from animals injected with HIV vector packaged from the parental pCMVΔR8.2 (A) or the multiply deleted pCMVΔR8.9 (B) constructs.



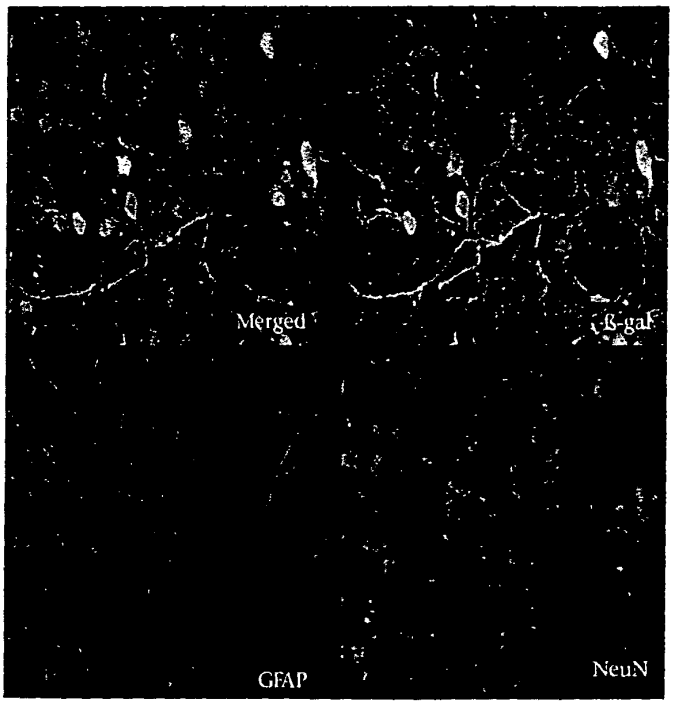


Figure 2. In vivo transduction of neurons by the multiply attenuated HIV vector. Confocal microscope images of a brain section from an animal injected with a multiply attenuated HIV vector expressing β-gal. Neurons, expressing NeuN (lower right), glial cells, expressing the glial fibrillary protein GFAP (lower left), and β-gal positive cells (upper right) are revealed by immunofluorescence. The merged image (upper left) shows that the majority of transduced cells are neurons.

In vitro gene transfer. The ability of deleted HIV-1 vector variants to tranfer genes into dividing and growth-arrested 293T cells was examined using luciferase as a reporter and a homologous MLV-based vector as a control. The deletion of *vif*, *vpr*, *vpu*, and *nef*, either individually or in combination, did not affect the ability of pseudotyped HIV vector particles to transduce both proliferating and γ-irradiated 293T cells (Table 1). As expected, MLV-mediated gene transfer was inhibited in growth-arrested cells. Similar results were obtained with a β-gal reporter construct (data not shown).

The efficient transduction of γ-irradiated 293T cells in the absence of the four accessory proteins of HIV-1 indicates that, in these cells, the determinants necessary for the nuclear transport of the HIV-1 preintegration complex are confined to the Gag and Pol polyproteins. However, in some other targets, additional karyophilic elements may be required. Vpr, for instance, promotes HIV nuclear import in terminally differentiated macrophages[16]. Correspondingly, HIV-derived vector particles produced in the absence of Vpr have a 50% decreased transduction efficiency in these cells[11]. Macrophages isolated from circulating monocytes from two healthy donors were inoculated in parallel with luciferase-expressing, VSV G-pseudotyped HIV-1 vectors produced from packaging constructs containing all HIV-1 genes besides *env* (pCMVΔR8.2), or lacking also either *vpr* (pCMVΔR8.3), *nef* (pCMVΔR8.4), or *vif*, *vpr*, *vpu*, and *nef* (pCMVΔR8.9). The results confirmed that HIV-mediated gene delivery to macrophages is reduced by approximately 50% in the absence of *vpr*. However, further deleting the other HIV-1 accessory genes had no additional negative effect (Table 2).

Table 1. Transduction efficiency* of VSV G-pseudotyped HIV-1 vectors variants in 293T cells.

	Env	Vif	Vpr	Vpu	Nef	Luciferase activity† (RLU) Dividing 293T	Irradiated 293T‡	Irrad./Div.§
pCMVΔR8.2	-	+	+	+	+	200,226±12,700	662,454±47,200	3.30
pCMVΔR8.3	-	+	-	+	+	151,727±31,650	449,234±101,500	2.96
pCMVΔR8.4	-	+	+	+	-	287,203±10,250	904,829±80,200	3.15
pCMVΔR8.5	-	-	-	+	+	194,152±5,450	660,799±105,250	3.40
pCMVΔR8.6	-	+	-	+	-	274,649±63,350	744,007±89,200	2.70
pCMVΔR8.7	-	-	-	+	-	339,800±9,450	706,200±35,500	2.07
pCMVΔR8.8	-	-	-	-	+	197,285±21,850	687,385±64,550	3.48
pCMVΔR8.9	-	-	-	-	-	293,091±39,950	786,680±162,350	2.68
MLV						246,679±35,760	9,214±324	0.03

*HIV-based vectors were produced from pHR'CAluc, the MLV-based vector from pCLCMVluc. Results for each packaging plasmids are based on duplicated transfections, duplicated infections with each supernatant and duplicated luciferase assays. †Activity per 1 ng of p24 for HIV vectors. ‡8,000 rad of γ-irradiation delivered by 3 minutes exposure to a ^{60}Co source §Higher levels of luciferase activity in irradiated cells transduced with HIV vectors likely reflect increased transcription efficiency in this setting, as described[16]. When using a β-gal expressing vector, ratio was approximately 1 when titer was determined by counting X-Gal positive cells.

Table 2. In vitro transduction of terminally differentiated macrophages.

Packaging plasmids	Luciferase activity (RLU)* Dividing 293T	Macrophages Donor A	Macrophages Donor B	Relative efficiency†
pCMVΔR8.2	160,647 (100%)	20,212 (100%)	21,809 (100%)	1
pCMVΔR8.3 (Δvpr)	121,795 (76%)	7,737 (38%)	7,937 (36%)	0.49
pCMVΔR8.4 (Δnef)	299,739 (186%)	34,774 (172%)	31,258 (143%)	0.85
pCMVΔR8.91 (Δvif Δvpr Δvpu Δnef)	208,559 (130%)	12,885 (63%)	11,642 (53%)	0.45
MLV	141,925 (88%)	130 (0.6%)	87 (0.4%)	0.006

*1 and 300 ng of 24 were used to transduce 293T cells and macrophages, respectively. †Mean transduction efficiency relative to pCMVΔR8.2 in macrophages (%) divided by the corresponding value in 293T cells. Average variability from two independent infections was less than 10%.

In vivo gene delivery. High-titer stocks of HIV-derived, VSV G-pseudotyped β-gal vectors made from packaging constructs containing (pCMVΔR8.2) or deleted in (pCMVΔR8.9) the *vif*, *vpr*, *vpu*, and *nef* genes were injected into the corpus striatum of the brain of adult rats. Two weeks later, the animals were sacrificed, and transduction rate was assessed by serial cryostatic sectioning and immunostaining of every sixth section for β-gal. The nature of the transduced cells was further determined by immunofluorescence costaining of representative sections with antibodies directed against β-gal and a neuron-specific marker. With both vectors, similarly high numbers of transduced cells, estimated at approximately 20,000 per animal, were visible within several millimeters of the injection site (Fig. 1). As previously observed with first generation HIV-1 vectors[11,12], the majority of these cells exhibited the morphological features of neurons and expressed the neuronal marker protein NeuN (Fig. 2).

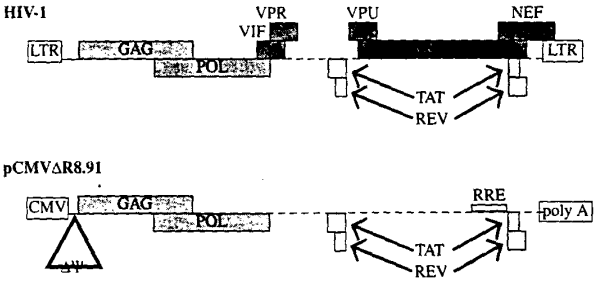


Figure 3. Comparison of the HIV-1 proviral DNA with the multiply deleted packaging plasmid pCMVΔR8.91.

Discussion

This work reveals that a multiply attenuated HIV-1 vector system in which five out the nine genes normally present in the parental virus have been deleted (Fig. 3) remains fully functional. The viral envelope as well as the four accessory proteins of HIV-1, Vif, Vpr, Vpu, and Nef, were indeed dispensable for the efficient generation of HIV-derived retroviral vector particles that could transduce nondividing cells in vitro, and deliver transgenes into nonmitotic targets such as terminally differentiated neurons in vivo.

These results can be partly explained by the particular cell type used to produce the vector. The requirement of HIV for Vif, a late viral protein that acts during assembly to promote the early steps of infection, is limited to virions produced from peripheral blood lymphocytes, macrophages, and some rare T lymphoid cell lines such as

H9, but is inapparent when particles are generated from COS, HeLa or 293 cells[17,18] (data not shown). Similarly, although Vpu was previously reported to stimulate the release of virions from T lymphoid, HeLa, or colonic carcinoma SW480 cells[19,20], we did not observe such an effect when producing particles from 293 or 293T cells.

Nef promotes the infectivity of HIV-1 virions and of HIV-1(amphotropic MLV) pseudotypes[21-25]. It was therefore surprising to find that HIV-derived retroviral vector particles, or HIV-1 virions[26] that are pseudotyped with the VSV G protein remain fully infectious in the absence of Nef. This does not reflect the higher relative infectivity of VSV G pseudotyped particles, because in the presence of limiting amounts of this envelope infectivity decreases to levels similar to that of non-pseudotyped HIV-1 virions, yet remains unaffected by Nef[6] (data not shown). These results rather suggest that the Nef requirement is restricted to virions penetrating cells by direct fusion at the plasma membrane, the major route of entry via the HIV and amphotropic MLV envelopes, while Nef is dispensable when this process occurs through receptor-mediated endocytosis and fusion in the endocytic compartment, the pathway targeted by the VSV G protein.

The lack of an absolute requirement for Vpr can be explained by the redundancy of mechanisms allowing for the nuclear transport of the HIV-1 preintegration complex, a process also governed by two other karyophilic vrial proteins, matrix (MA) and integrase (IN)[8-10,27]. When all three viral proteins are functional, Vpr seems to play only an additive role, visible mainly in terminally differentiated macrophages (Tables 1 and 2)[10,11,16]. The present data demonstrate that, at least for delivering transgenes in the central nervous system, Vpr is completely dispensable. Nevertheless, the requirements for transduction of cells such as myocytes, hepatocytes, or hematopoietic progenitors might differ, so that the issue will have to be addressed for each one of the potential targets of HIV-based vectors. Of note, the development of stable packaging cell lines will be greatly facilitated if Vpr, a strong inducer of cell cycle arrest[28,29], is not required.

The combined removal of five genes eliminates the possibility that pathogenic HIV-1 recombinants might arise during the preparation of HIV-derived vectors, as these genes encode viral proteins that either have been proven or are strongly suspected to represent crucial virulence factors. Through the recognition of CD4, the HIV envelope dictates the tropism of this virus for T lymphocytes and macrophages, thereby conditioning the modalities of HIV-induced disease. Nef also plays a pivotal role in the in vivo replication of primate lentiviruses and in AIDS pathogenesis. In at least a subset of long-term survivors of HIV infection, the absence of disease progression appears to reflect infection with *nef*-defective strains[30,31]. Similarly, adult rhesus monkeys inoculated with a *nef*-deleted SIV exhibit only low levels of viremia, and not only remain asymptomatic[32] but also become resistant to subsequent challenges with wild-type SIV, leading to the suggestion that *nef*-mutated viruses could constitute the basis for live-attenuated AIDS vaccines[33,34]. The *vif* gene of both HIV and SIV is essential for growth in PBL and macrophages, the natural cellular targets for these viruses[17,18,35,36]. Vpr also promotes viral replication in vivo, as indicated by the rapid emergence of revertants in monkeys inoculated with *vpr*-defective SIV mutants[37]. In HIV-1, Vpr probably plays a role that is even more essential, since it fulfills, in this virus, the functions of Vpr and Vpx in SIV[38]. Correspondingly, HIV-1 strains with mutations in *vpr* were isolated from a long-term nonprogressing mother and her infected child[39]. Finally, Vpu is likely to be equally crucial in view of its high degree of conservation among HIV-1 isolates.

We suggest that the degree of biosafety of the HIV-derived vectors described here, once they are built into stable packaging cell lines, will not be fundamentally different from that of MLV-based vectors currently used in human clinical trials. Because lentiviral vectors have capabilities that are far superior to those of oncoretroviral vectors, our results thus open new avenues for human gene therapy.

Experimental protocol

DNA constructions. The plasmid pCMVΔR8.2 was described previously (11, 12). In CMVΔR8.3, a frame shift mutation was introduced in the Vpr coding sequence by blunting a unique EcoRI site. pCMVΔR8.5 (ΔVifΔVpr) was constructed by deleting the sequence between the 3′ end of the *pol* gene and this EcoRI site. pCMVΔR8.6 (ΔVprΔNef) was obtained by blunting the XhoI site in the Nef coding region of pCMVΔR8.3. pCMVΔR8.7 (ΔVprΔVifΔNef) was obtained by deleting the sequence between the env stop codon and the XhoI site of pCMVΔR8.5. To generate pCMVΔR8.9 (ΔVprΔVifΔVpuΔNef), a 156-nt Ssp1-NotI fragment was deleted in pCMVΔR8.7. In pCMVΔR8.91 the piece of *nef* that remained in pCMVΔR8.9 was deleted. pCMVΔR8.8 (ΔVprΔVifΔVpu) was obtained by replacing the [SalI-BamHI] fragment of pCMVΔR8.5 with the corresponding region of pCMVΔR8.9. pCMVΔR8.4 (ΔNef) was obtained by replacing the [BamHI-XbaI] fragment of pCMVΔR8.2 with the equivalent fragment from pCMVΔR8.9. All mutations and deletions were verified by DNA sequence analysis.

Preparation of high-titer vector stocks. High-titer stocks of lentiviral vectors carrying a CMV-driven LacZ gene, packaged with either wild-type or multiply attenuated HIV-derived constructs and pseudotyped with VSV G envelope, were prepared by transient transfection of 293T cells as previously described[11,12] and stored at -80°C. Prior to injection, vectors were resuspended by slow vortexing 4 hrs at room temperature, and adjusted to a p24 concentration of 1.5 mg/ml, corresponding to a titer of 1.8×10^9 transducing units/ml on HeLa. Vector stocks were tested for the absence of replication-competent HIV-derived virus as described[11,12].

In vitro transduction. All in vitro transductions were done in 6-well plates[11,12]. Multiplicity of infection for 293T cells was approximately 0.01 when 1 ng of p24 was used, as determined with a β-gal expression vector. Macrophages were isolated from the peripheral blood of healthy donors by attachment to bacterial petri dishes coated with human IgG. Adherent cells were cultured in RPMI +10% FCS in a 5% CO_2 atmosphere. After 1 week, attached cells were trypsinized briefly, scraped from the plastic, transferred to 6 tissue culture–treated 6-well plates, and cultured for one more week before inoculation with 500 µl of vector-containing supernatant (300 ng of p24 equivalent for the HIV-based vector). 293T cell were transduced in parallel with 1 ng of p24 equivalent. Proteins were extracted for the luciferase assay 72 h postinfection. Luciferase and β-gal activities were detected as previously described[11,12].

In vivo gene delivery. Eight-week-old Fischer 344 male rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and maintained under published NIH guidelines. All surgical procedures were performed with the rats under isofluorane gas anesthesia and using aseptic procedures, according to described protocols[11,12]. Six animals were injected for each vector type, and the contra lateral side of one from each group was injected with HNE vehicle only as control. Two weeks after vector injection the animals were sacrificed and their brain examined as previously described[11,12]. β-gal immunoreactive cells were counted for every sixth section of the corpus striatum, and plotted in antero-posterior sampling order for each individual animal. No cell with similar immunoreactivity was ever observed in the contra lateral side of the brain either uninjected or injected with vehicle only, nor in numerous other animals injected with homologous MLV-derived vectors. Representative coronal sections from each experimental group were analyzed by confocal microscopy following immunofluerescence staining with a mixture of rabbit anti-β-gal (1:5000; Cortex, Irvine, CA), guinea pig anti-glial fibrillary acidic protein (GFAP, 1:250, Chemicon), and mouse anti-NeuN (1:500; Chemicon, Temecula, CA) antibodies, as described[11,12].

Acknowledgments

We thank Leslie Barden for the artwork. RZ is the recipient of a fellowship from the Swiss National Science Foundation. This work was supported by grants from the National Institutes of Health and from the HN and Frances Berger Foundation to DT.

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